Biometals (2012) 25:893–903
DOI 10.1007/s10534-012-9556-4

Physiological metal uptake by *Nostoc punctiforme*

L. Hudek · S. Rai · A. Michalczyk · L. C. Rai · B. A. Neilan · M. Leigh Ackland

Received: 20 December 2011 / Accepted: 1 May 2012 / Published online: 17 May 2012

Abstract Trace metals are required for many cellular processes. The acquisition of trace elements from the environment includes a rapid adsorption of metals to the cell surface, followed by a slower internalization. We investigated the uptake of the trace elements Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} and the non-essential divalent cation Cd^{2+} in the cyanobacterium *Nostoc punctiforme*. For each metal, a dose response study based on cell viability showed that the highest non-toxic concentrations were: 0.5 μM Cd^{2+}, 2 μM Co^{2+}, 0.5 μM Cu^{2+}, 500 μM Mn^{2+}, 1 μM Ni^{2+}, and 18 μM Zn^{2+}. Cells exposed to these non-toxic concentrations with combinations of Zn^{2+} and Cd^{2+}, Zn^{2+} and Co^{2+}, Zn^{2+} and Cu^{2+} or Zn^{2+} and Ni^{2+}, had reduced growth in comparison to controls. Cells exposed to metal combinations with the addition of 500 μM Mn^{2+} showed similar growth compared to the untreated controls. Metal levels were measured after one and 72 h for whole cells and absorbed (EDTA-resistant) fractions and used to calculate differential uptake rates for each metal. The differences in binding and internalisation between different metals indicate different uptake processes exist for each metal. For each metal, competitive uptake experiments using ^{65}Zn showed that after 72 h of exposure Zn^{2+} uptake was reduced by most metals particularly 0.5 μM Cd^{2+}, while 2 μM Co^{2+} increased Zn^{2+} uptake. This study demonstrates that *N. punctiforme* discriminates between different metals and favourably substitutes their uptake to avoid the toxic effects of particular metals.

Keywords Cyanobacteria · Divalent cations · Metal uptake · Adsorption · Absorption · Bioremediation

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Introduction

Cyanobacteria are photosynthetic prokaryotes, proposed to have evolved over 3.5 billion years. They have contributed to the evolution of higher organisms through endosymbiotic events and to the earth's environment through generation of oxygen. Their persistence indicates their capacity to tolerate or adapt to a wide range of environmental stressors. Cyanobacteria provide a good model for analysing the mechanisms involved in tolerance and adaption to

environmental change. Some species of cyanobacteria have been shown to accumulate heavy metals and have consequently received interest as a tool for bioremediation, particularly for the sorption of metals from contaminated wastewater. These species include *Anabaena nodosum* (Chong and Volesky 1996), *Nostoc linkia* (El-Enany and Issa 2000), *Microcystis aeruginosa* (Pradhan et al. 1998), and *Synechococcus* sp. PCC7942 (Gardea-Torresdeya et al. 1998) among others (Mehta and Gaur 2005).

The capacity of cyanobacteria to adsorb metals is a function of their cell surface that consist of complex structures in distinct layers each with unique molecular functional groups and metal binding properties (Yee et al. 2004). Most commonly, the external cell wall is covered by S-layers comprising two-dimensional crystalline arrays of a single species of glycoprotein that covers the entire cell surface, beneath which lies the outer membrane, the peptidoglycan layer, and the cytoplasmic membrane (Hoiczyk and Hansel 2000). Extracellular secreted polysaccharides (exopolysaccharides) of Nostoc have also been identified as having a high biosorption capacity for chromium (Sharma et al. 2008). Initial metal adsorption to these cell surfaces occurs rapidly (Mehta and Gaur 2005). The rapid binding of metals on to cyanobacterial cell surface occurs as a result of negatively charged groups on membrane and in extracellular polymeric substances (EPS) while the intracellular levels are maintained via (i) metal exclusion from cells (ii) metal chelation by phytochelatin and metallothioneins (iii) sequestration in polyphosphate bodies (Dohnalkova et al. 2005; Mehta and Gaur 2005). The delicate balance between metal requirement and toxic over exposure such as oxidative damage, is maintained through tight regulation of uptake and trafficking through the filter layers of EPS and membrane and further its detoxification and sequestration (Dohnalkova et al. 2005; Mehta and Gaur 2005).

Many studies have established the ability of different cyanobacterial cells to sorb metal ions, including Cd^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} (Azeez and Banerjee 1991; Gardea-Torresdeya et al. 1998; Awasthi and Rai 2006; Baptista and Vasconcelos 2006; Scholnick and Keren 2006). In these studies, *Synechocystis* sp., *Anacystis nidulans*, and *Spirulina platensis* have been exposed to an array of metal concentrations (μM to M) over various time periods (minutes–days) in differing treatment media solutions. However, in these studies the concentration of metals is variably reported as ppm, M, or mg/L, making comparisons between treatment concentrations difficult (Azeez and Banerjee 1991; Gardea-Torresdeya et al. 1998; Scholnick and Keren 2006). These studies indicated a range of sensitivity of many cyanobacteria species to heavy metals.

A major function of the cyanobacterial cell wall is to facilitate transport of nutrients and metabolites (Hoiczyk and Hansel 2000). Substantial data exists showing sorption of metals in cyanobacteria, the capacity of live cells to tolerate or accumulate metals is relatively unknown. *Nostoc punctiforme*, for example, is quite sensitive to low concentrations (22 μM) of Zn^{2+} (Hudek et al. 2009).

It is essential for cell viability for trace levels of metals to infiltrate the cell wall and become internalized. Internalized metals are cofactors for processes such as Cu^{2+} in thylakoidal plastocyanin and cytochrome c oxidase, Zn^{2+} in carboxysomal carbonic anhydrase, Co^{2+} in cobalamine, Mn^{2+} in thylakoidal water splitting oxygen evolving complex (Miccadei and Floridi 1993; Ybarra and Webbb 1999; Cavet et al. 2003; Choudhary et al. 2006; Tanioka et al. 2009). For metals to be internalized (imported) cyanobacteria have evolved unique metal transport systems (Ma et al. 2009). Porins embedded in the outer membrane facilitate nonselective passive diffusion of metal ions across the outer membrane, while active transport systems operate both in the outer and inner membranes (Ma et al. 2009). In this study, we investigated the total sorption of metals by whole live *N. punctiforme* cells, the adsorption of metals to the cell surface (EDTA-sensitive) and the absorption into an EDTA-resistant compartment that represents the internalized metals. *Nostoc punctiforme* provides a good model for investigating the mechanisms of metal binding, uptake, sequestration and efflux, as it grows as a free-living organism that is differentially responsive to metals. Furthermore, it is simpler than eukaryotic systems as it does not have intracellular organelles that may sequester metals.

Materials and methods

Cell culture

Stock cultures of *Nostoc punctiforme* PCC73120 cells were cultured as previously described (Anderson et al. 2006; Hudek et al. 2009). Experiments involving

metal treatments were conducted in 200 mL conical flasks containing 25 mL of culture media under previously described conditions (Hudek et al. 2009).

Viable cell counts

Cell viability was measured using Trypan Blue exclusion (Asayama et al. 2004; Qiu and Hogstrand 2005; Hudek et al. 2009). Numbers of live and dead cells were recorded. Cell viability was measured at intervals of 24 h over a seven-day period. This was used to establish the effect of metal regimes for all treatments.

Metal treatments

Stock solutions of 100 mM of each Cd^{2+} ($3CdSO_4$), Co^{2+} ($CoCl_2$), Cu^{2+} ($CuSO_4$), Mn^{2+} ($MnCl_2$), Ni^{2+} ($NiCl_2$), and Zn^{2+} ($ZnCl_2$) were prepared. Aliquots of the metal stock solutions were added into BG11 medium and the pH adjusted to 7.8. Triplicates of 25 mL cultures containing an initial wet weight of 100 mg of cells (4 mg of cells/mL giving a consistent optical density of 0.25 and a dry weight of 25 mg) were grown in metal concentrations ranging from 0.1 to 1000 μM over a seven-day period. For comparison, controls cells were grown in media without added metals.

Mixed metal treatments were prepared as described above and contained metal concentrations at levels shown to have no significant impact on cell growth for each metal (0.5 μM Cd^{2+}, 2 μM Co^{2+}, 0.5 μM Cu^{2+}, 500 μM Mn^{2+}, 1 μM Ni^{2+}, and 18 μM Zn^{2+} in BG11).

The speciation and levels of free metals in all media solutions were predicted using MINEQL+ software (4.5). Results from MINEQL+ indicated that there was less then a 15 % reduction (>85 % bioavailability) in any of the free metals at pH 7.8 for all media treatment solutions. This reduction in free metals is consistent with reported free metal reductions at a pH of 7.8 (Qiu and Hogstrand 2005). A constant pH of 7.8 was maintained in experiments, providing optimum conditions for cell growth, without favoring a particular metal treatment.

Quantification of intracellular and extracellular metals using atomic absorption spectroscopy

The relative rate of accumulation of each metal was measured in triplicate cultures grown with the addition of each individual metal at prescribed concentrations (Table 1). Cells were treated for 0, 1, and 72 h with 0 μM (control) or 0.5 μM Cd^{2+}, 2 μM Co^{2+}, 0.5 μM Cu^{2+}, 500 μM Mn^{2+}, 1 μM Ni^{2+}, and 18 μM Zn^{2+} (corresponding to <0.001 ppm or 0.2, 0.1, 0.1, 67.1, 0.2, and 0.6 ppm of Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, respectively). To collect both adsorbed and intracellular metal, each treatment group was divided into two groups, half of which were treated with 1 mL of 20 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0, for 15 min prior to harvesting (to remove adsorbed Zn^{2+}). The remainder were harvested immediately after treatment and contained the total amount of cell-associated metal (Hudek et al. 2009). Following EDTA treatment, cells were centrifuged for 10 min at 2,500 *g*. Media (1.5 mL) and EDTA treated and untreated cells were collected for further analysis. EDTA and concentrated nitric acid were tested for purity and were all below $<1.0 \times 10^{-8}$ ppm (or below detection limit). Cells (3×10^5) from each treatment (±EDTA) were collected and dried in a heating block at 60 °C for 8 h. Cell pellets were digested in concentrated nitric acid for 2 h, then analysed for individual metals using flame atomic absorption spectroscopy (AAS).

Table 1 Highest metal concentrations that did not reduce cell growth over 7 days of exposure based on Trypan blue exclusion experiments

Metal	Concentration (μM)
Control	0
Cd^{2+}	0.5
Co^{2+}	2
Cu^{2+}	0.5
Mn^{2+}	500
Ni^{2+}	1
Zn^{2+}	18

Listed concentrations were used for metal treatments in all experiments

Quantification of intracellular and extracellular metals using inductively coupled plasma mass spectrometry

Triplicate cultures of 100 mg of cells in 25 mL of media were exposed for 0, 1, and 72 h to a solution

containing no metals (0 μM, control) or to a mixed solution containing Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} at prescribed levels (Table 1). Samples representing each metal treatment were divided into two groups, one of which was treated with 1 mL of 20 mM EDTA as described above. The EDTA and concentrated nitric acid were tested for purity, indicating metal levels were all below 1.0×10^{-8} ppm. Cells (3×10^5) from each metal treatment were collected and dried in a heating block at 60 °C for 8 h or until the pellets weighed ~25 mg. Cell pellets were digested in nitric acid, as described for AAS, and analysed for each metal simultaneously using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Physiological and competitive metal uptake using ^{65}Zn

For uptake experiments, cell pellets (100 mg) were prepared in triplicate for 0, 1, and 72 h treatments. Each sample was divided into two sets, each containing 1 mL of 0 μM metal (control), 0.5 μM Cd^{2+} + 18 μM Zn^{2+}, 2 μM Co^{2+} + 18 μM Zn^{2+}, 0.5 μM Cu^{2+} + 18 μM Zn^{2+}, 500 μM Mn^{2+} + 18 μM Zn^{2+}, 1 μM Ni^{2+} and 18 μM Zn^{2+}, in BG11 medium. One set had 0.5 μCi/mL ^{65}Zn (Oak Ridge National Laboratory) added to the culture medium while the other set was used for protein assay. Cells from each treatment group were centrifuged at 8,000 *g* for 2 min then rinsed with 1 mL of 20 mM EDTA. Centrifuged cell pellets, culture medium and the EDTA rinse were retained and counts per minute of ^{65}Zn were obtained (Perkin Elmer 1480 Automatic Gamma Counter). For non-radiolabelled cells, the total protein was measured (Pierce BCA protein assay kit) (Ackland and McArdle 1996; Ran et al. 2007). For the ^{65}Zn-treated cells, the pmol Zn^{2+}/μg of protein was calculated for each time point, for both adsorbed and internalized Zn^{2+} (Ackland and McArdle 1996).

Statistical methods

Statistical methods were based on normally distributed data collected from all experiments with three independent samples for each experiment. Data sets were all firstly produced in Microsoft Excel then converted to the appropriate graph type for viability tests, AAS, ICP-MS and physiological competitive. The statistical computer programme SPSS (v17.0 for Windows) was used for all statistical analyses. Probability plots (P–P Plots) were produced for all data sets to test for normal distribution. Statistical tests included one-way analysis of variance (one-way ANOVA) and Tukey's honest significant difference test for the growth curve, AAS, ICP-MS and physiological competitive uptake data. All statistical analyses was tested against the probability value (*p*-value) of <0.05.

Results

Divalent cations differentially affect *N. punctiforme* viability

The tolerance of *N. punctiforme* to different metals (Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}) was established by exposing cells to increasing metal concentrations ranging from 0 to 1000 μM for 7 days. Viable cell numbers for each metal concentration were measured using Trypan Blue exclusion tests (Asayama et al. 2004; Qiu and Hogstrand 2005; Hudek et al. 2009). Concentrations of Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} exceeding 0.5 μM Cd^{2+}, 2 μM Co^{2+}, 0.5 μM Cu^{2+}, 500 μM Mn^{2+}, 1 μM Ni^{2+}, and 18 μM Zn^{2+} caused a significant ($p < 0.05$) decline in the number of viable cells present after 7 days (Fig. 1a–f). In contrast, cells exposed to metal concentrations below these levels showed no significant decrease in viable cell number compared to the control (Fig. 1a–f). The highest metal concentrations that did not reduce cell viability were used as the baseline metal levels for successive experiments (Table 1).

To investigate the effect of metal competition, cells were exposed to combinations of metals. Cells exposed to each metal at the highest concentration that did not reduce cell viability in the added presence of 18 μM Zn^{2+} for 7 days also showed a significant reduction in total cell counts relative to the control (Table 1) (Fig. 2). In contrast to this, however, there was no significant difference ($p < 0.05$) in viable cell counts when 500 μM Mn^{2+} was added to the cultures containing mixtures of Zn^{2+} and each metal (Fig. 2). When cells were treated with a solution containing multiple metals (18 μM Zn^{2+}, 0.5 μM Cd^{2+}, 2 μM Co^{2+}, 0.5 μM Cu^{2+}, and 1 μM Ni^{2+}) in addition to the presence of 500 μM Mn^{2+} no significant difference in cell growth was seen over 7 days, relative to the control cells (Fig. 2).

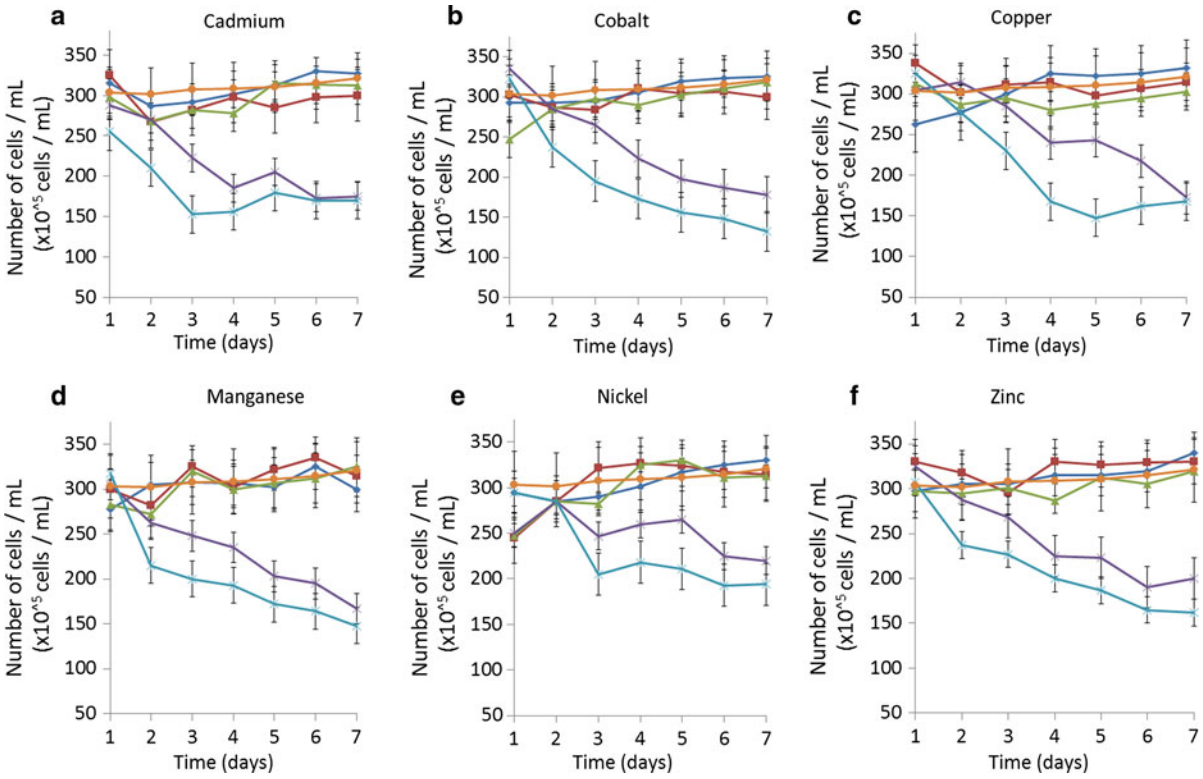

Fig. 1 Highest viable concentration of (**a**) Cd^{2+} (0 μM added Cd^{2+}, *open circle*) (0.05 μM Cd^{2+}, *open diamond*) (0.1 μM Cd^{2+}, *open square*) (0.5 μM Cd^{2+}, *open triangle*) (1 μM Cd^{2+}, *astrick*) (2 μM Cd^{2+}, *times symbol*) (**b**) Co^{2+} (0 μM added Co^{2+}, *open circle*) (0.5 μM Co^{2+}, *open diamond*) (1 μM Co^{2+}, *open square*) (2 μM Co^{2+}, *open triangle*) (4 μM Co^{2+}, *astrick*) (8 μM Co^{2+}, *times symbol*) (**c**) Cu^{2+} (0 μM added Cu^{2+}, *open circle*) (0.05 μM Cu^{2+}, *open diamond*) (0.1 μM Cu^{2+}, *open square*) (0.5 μM Cu^{2+}, *open triangle*) (1 μM Cu^{2+}, *astrick*) (2 μM Cu^{2+}, *times symbol*) (**d**) Mn^{2+} (0 μM added Mn^{2+}, *open circle*) (100 μM Mn^{2+}, *open diamond*) (250 μM Mn^{2+}, *open square*) (500 μM Mn^{2+}, *open triangle*) (750 μM Mn^{2+}, *astrick*) (1000 μM Mn^{2+}, *times symbol*), (**e**) Ni^{2+} (0 μM added Cd^{2+}, *open circle*) (0.1 μM Ni^{2+}, *open diamond*) (0.5 μM Ni^{2+}, *open square*) (1 μM Ni^{2+}, *open triangle*) (2 μM Ni^{2+}, *astrick*) (4 μM Ni^{2+}, *times symbol*) and (**f**) Zn^{2+} (0 μM added Zn^{2+}, *open circle*) (6 μM Zn^{2+}, *open diamond*) (12 μM Zn^{2+}, *open square*) (18 μM Zn^{2+}, *open triangle*) (24 μM Zn^{2+}, *astrick*) (30 μM Zn^{2+}, *times symbol*) for *N. punctiforme* was established using trypan blue exclusion tests, indicating viable cell numbers

Individual divalent cations are accumulated differentially by *N. punctiforme*

N. punctiforme cells were treated for one and 72 h with each metal individually at the prescribed concentrations (Table 1). Quantification of the total cellular (whole cell) metal levels showed that cells accumulated each metal at different rates ranging from 0.2 pmol/μg total protein for Cd^{2+} to 341 pmol/μg protein for Mn^{2+}. Total metal levels after 1 h exposure were 0.2, 4, 2, 341, 1.4, and 5 pmol/μg protein for Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, respectively (Table 2). A significantly ($p < 0.05$) greater proportion of each metal was in the whole cells compared with the EDTA-resistant (internalized) fraction for all treatments after 1 h (Table 2). After 1 h exposure to the individual metals, EDTA-resistant levels of 0.02, 3, 1.4, 29, 0.4, and 2 pmol/μg total protein were found for Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, respectively (Table 2). After 72 h of exposure, higher levels of heavy metals were found in the whole cell compared to the EDTA-resistant level for all treatments (Table 2). At 72 h significantly more total cellular Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} was detected compared to after 1 h, however, the amount of total Cu^{2+} taken up at 72 h was similar to that after 1 h (Table 2). Total metal sorbed by whole cells compared to that internalized into an EDTA-resistant fraction were lower for Cd^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} after 72 h in comparison to 1 h levels, while Mn^{2+} and Zn^{2+} increased significantly after 72 h (Table 2). After 1 h, there was a nine- and 12-fold more Cd^{2+}

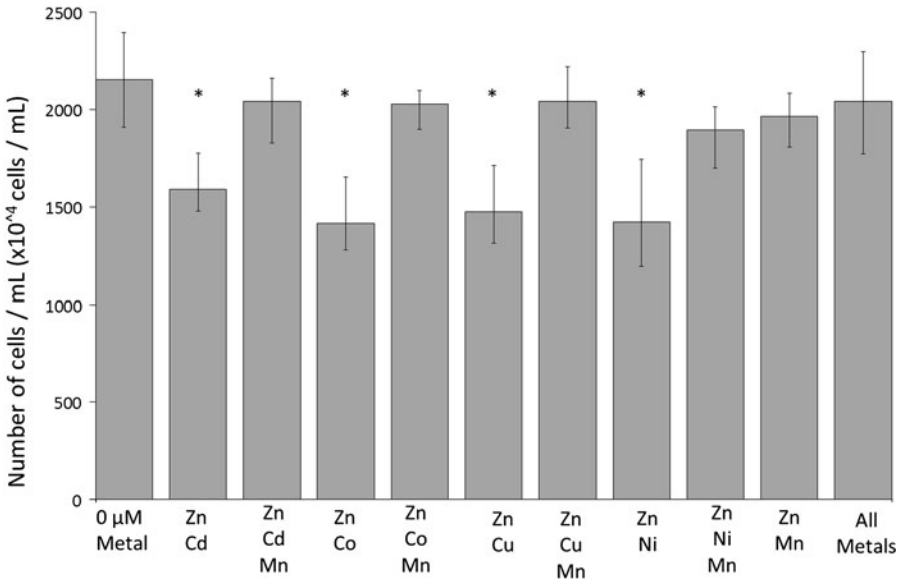

Fig. 2 Trypan blue exclusion tests for establishing effects of multiple metal exposure regimes based on viable cell numbers. Significant difference ($p < 0.05$) between number of cells/mL as a result of metal exposure regime and compared to no metal treatment (control) is denoted by *asterisk* (*)

and Mn^{2+}, respectively for the whole cell compared to internalized EDTA-resistant fractions (Table 2). Mn^{2+} levels were the highest after 1 h for both whole cell and EDTA-resistant (internalized) fractions at 341 and 29 pmol/µg of protein (Table 2). After 72 h Mn^{2+} levels increased to 699 and 47 pmol/µg protein for total cell and EDTA-resistant levels (Table 2). Total cell to EDTA-resistant levels also increased from 12 to 15 after 72 h exposure to 500 µM Mn^{2+} (Table 2). The total cellular Zn^{2+} level was 31-fold higher then the internalized fraction after 72 h, with an increase in bound Zn^{2+} from 3 to 45 pmol/µg of protein and a reduction of internalized from 2 to 1.5 pmol/µg of protein (Table 2).

Individual metal cations are accumulated differentially from a mixed metal solution

Metal levels for whole cells and EDTA-resistant and EDTA-sensitive fractions were quantified for cells treated with a mix of the highest concentration of each metal that did not reduce cell viability was carried out using ICP-MS (Table 1). These results showed that significantly more metal was in the whole cells compared with the EDTA-resistant fractions for Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} after 1 h of treatment 2, 0.8, 0.4, 214, 2, and 5 pmol/µg of total protein, respectively, compared to 1, 0.4, 0.2, 37, 0.6, and 4 pmol/µg of protein, respectively (Table 3). After 72 h Co^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} treatments contained 2, 3, 447, 30, and 20 pmol/µg of protein, respectively, for the whole cells, significantly more than the EDTA-resistant fractions with 1.7, 0.6, 49, 0.8, and 3 pmol/µg of protein, respectively (Table 3).

After 72 h total cellular (whole cell) Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} increased significantly to 3, 0.6, 447, 25, and 20 pmol/µg of protein from the 1 h levels of 0.8, 0.4, 214, 2, and 5 pmol/µg of protein, respectively (Table 3). After 72 h of treatment internalized Cd^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+} significantly increased from 1, 0.4, 0.2, and 37 to 1.7, 0.6, 0.7, and 49 pmol/µg of protein, respectively (Table 3). After 72 h treatment with the mixed metal media the internalized level of Ni^{2+} was the same as after 1 h while the Zn^{2+} level decreased significantly from 4 to 3 pmol/µgram of protein (Table 3).

The level of total sorbed metal compared to the internalized EDTA-resistant fraction reduced significantly after 72 h in comparison to the 1 h levels for Co^{2+} (0.5–0.2) and Zn^{2+} (0.8–0.2-fold reduction) (Table 3). Levels of total sorbed metal compared to the internalized EDTA-resistant fraction increased significantly after 72 h for Cd^{2+} and Cu^{2+} from 0.5 and 0.6 to 0.9, and 1.1, respectively (Table 3).

Divalent cations have varying degrees of competition for accumulation by *N. punctiforme*

To determine the effect different heavy metals have on Zn^{2+} uptake, *N. punctiforme* cells were exposed to

Table 2 Total metal content for whole cells and EDTA-resistant amounts (pmol/μg of protein) after 1 and 72 h exposure to individual metals

Metal levels for whole cell, EDTA-sensitive and EDTA-resistant (pmol/μg of protein)

1 h							72 h						
	Cd^{2+}	Co^{2+}	Cu^{2+}	Mn^{2+}	Ni^{2+}	Zn^{2+}		Cd^{2+}	Co^{2+}	Cu^{2+}	Mn^{2+}	Ni^{2+}	Zn^{2+}
Whole cell	0.2	4	2	341	1.4	5	Whole cell	0.3	6	2	699	4	46
	± > 0.1	±0.3	±0.2	±7	±0.2	±1		± > 0.1	±0.6	±0.7	±16	±0.8	±9
EDTA sensitive	0.2	1	0.6	312	1	3	EDTA sensitive	0.1	1	0.001	652	2	45
	± 0.2	±0.3	± > 0.01	±2	± > 0.01	±0.3		± > 0.01	± > 0.01	± > 0.01	±7	± > 0.01	±0.2
EDTA resistant	0.02	3	1.4	29	0.4	2	EDTA resistant	0.2	5	2	47	2	1.5
	± > 0.1	±0.3	±0.1	±3	± > 0.1	±1		± > 0.1	±0.3	±0.2	±11	±0.2	±0.6
Fold difference	9	1.3	2	12	4	3	Fold difference	1.3	1.1	1.2	15	2	31

The fold difference between EDTA-resistant fraction (internalized) and metal in whole cell for individual metal treatments after 1 and 72 h is also shown

18 μM Zn^{2+} with ^{65}Zn in the presence or absence of Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, or Ni^{2+} (Table 1). ^{65}Zn levels in EDTA-treated cell pellets were measured after 1 and 72 h (Fig. 3a and b). After exposure to the metal combinations 0.5 μM Cd^{2+} + 18 μM Zn^{2+}, 0.5 μM Cu^{2+} + 18 μM Zn^{2+}, 1 μM Ni^{2+} + 18 μM Zn^{2+}, 2 μM Co^{2+} + 18 μM Zn^{2+}, 500 μM Mn^{2+} + 18 μM Zn^{2+} and the mixed metal solution (MMS) (0.5 μM Cd^{2+}, 2 μM Co^{2+}, 0.5 μM Cu^{2+}, 500 μM Mn^{2+}, 1 μM Ni^{2+}, and 18 μM Zn^{2+}) the Co^{2+}, Cu^{2+}, and Ni^{2+} containing co-metal solutions and the MMS total Zn^{2+} accumulation and the level of EDTA-resistant Zn^{2+}, was significantly reduced to 0.74, 0.69, 0.54, and 0.53 pmol per μg/protein, respectively, in comparison with the Zn^{2+} only treatment (0.79 pmol/μg protein) (Fig. 3a).

The accumulation of Zn^{2+} was significantly higher after 72 h for all treatments except for the Zn^{2+} + Cd^{2+} treatment, where the amount was significantly reduced in comparison with 1 h levels (0.77–0.32 pmol/μg protein) (Fig. 3a, b). After 72 h the Co^{2+} + Zn^{2+} treatment contained 2.9 pmol/μg of protein, significantly more Zn^{2+} then for the Zn^{2+} only treatment (2.4 pmol/μg of total protein), indicating Co^{2+} significantly enhanced Zn^{2+} uptake under these conditions (Fig. 3b).

Discussion

The bioaccumulation of both individual and multiple metals was investigated in *N. punctiforme*. For each metal, the highest concentration that did not reduce the cell viability over 7 days was established by quantifying the number of viable cells for individual exposures (Fig. 1a–f). These findings showing the relative toxicity of Cd^{2+}, Co2 + , Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, indicate that there is a wide variation in relative toxicity for different metals in *N. punctiforme*. These levels provided a baseline for subsequent experiments investigating metal uptake by *N. punctiforme*.

Cyanobacteria are particularly tolerant to high Mn^{2+} concentrations, seen in previous studies where concentrations exceeding 1.8 mM (99 mg/mL) were non-toxic in mixed cyanobacterial mats (Bender et al. 1994; Bartsevich and Pakrasi 1996). This is 3.5 times higher then the highest concentration used in the present study, where 500 μM of Mn^{2+} (27 mg/mL)

Table 3 Total metal content for whole cell and EDTA-resistant amount fraction (pmol/μg of protein) after 1 and 72 h exposure to a mixed metal solution of 0.5 μM Cd^{2+}, 2 μM Co^{2+}, 0.5 μM Cu^{2+}, 500 μM Mn^{2+}, 1 μM Ni^{2+}, and 18 μM Zn^{2+} in BG11 after 1 h and 72 h

Metal levels for whole cell, EDTA-sensitive and EDTA-resistant (pmol/μg of protein)													
1 h							72 h						
	Cd^{2+}	Co^{2+}	Cu^{2+}	Mn^{2+}	Ni^{2+}	Zn^{2+}		Cd^{2+}	Co^{2+}	Cu^{2+}	Mn^{2+}	Ni^{2+}	Zn^{2+}
Whole cell	2	0.8	0.4	214	2	5	Whole cell	2	3	0.6	447	3	20
	±0.2	±0.2	±0.08	±4	±0.3	±0.6		±0.2	±0.2	± > 0.01	±6.7	±0.3	±1.7
EDTA sensitive	1	0.4	0.2	177	1.4	1	EDTA sensitive	0.3	2.4	0.001	398	2.2	17
	±0.2	±0.2	±0.07	± 52	±0.4	±0.2		±0.02	±0.3	± > 0.01	±7	±0.03	±0.5
EDTA resistant	1	0.4	0.2	37	0.6	4	EDTA resistant	1.7	0.6	0.7	49	0.8	3
	±0.2	± > 0.01	±0.06	±2.3	±0.1	±0.3		±0.02	±0.07	± > 0.01	±8	±0.2	±0.2
Fold difference	0.5	0.5	0.6	0.2	0.3	0.8	Fold difference	0.9	0.2	1.1	0.1	0.3	0.2

The fold difference between EDTA-resistant fraction (internalized) and metal in whole cell for individual metal treatments after 1 and 72 h is also shown

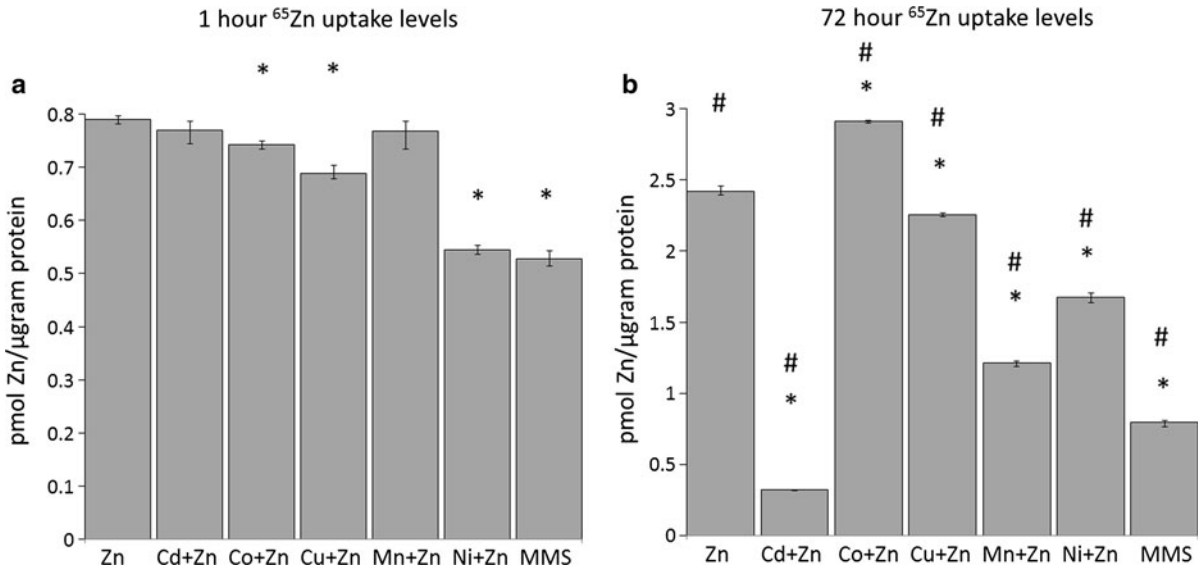

Fig. 3 Competitive ^{65}Zn uptake analyses for Zn^{2+} against Zn^{2+} and Cd^{2+}, Zn^{2+} and Co^{2+}, Zn^{2+} and Cu^{2+}, Zn^{2+} and Mn^{2+}, Zn^{2+} and Ni^{2+} individually and in a mixed solution containing all metals (Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} in BG11 medium) after 1 h (**a**) and 72 h (**b**) of exposure. Significant difference ($p < 0.05$) between metal concentrations for metal treatments in comparison with to no metal treatment (control) is denoted by *asterisk* (*). Significant difference ($p < 0.05$) between 1 h and 72 h metal concentrations for metal treatments is denoted by *hash* symbol (#)

was the highest dose that did not reduce the cell viability for *N. punctiforme* (Fig. 1d). The low toxicity of Mn^{2+} may relate to its requirement as an essential cofactor for electron transfer during photosynthesis (Scholnick and Keren 2006). Internalized Mn^{2+} was lower than the adsorbed levels, however overall Mn^{2+} levels were still much higher than for the other metals (Tables 2 and 3). The capacity of *N. punctiforme* to tolerate high levels of Mn^{2+} (500 μM), particularly in the presence of other metals, indicates its potential as a bioremediating microorganism in Mn^{2+}-contaminated sites and, due to its symbiotic nature (with lichens and plants), for rhizoremediation (Meeks et al. 2001; Wu et al. 2006; Hameed and Ebrahim 2007). Previous

studies identified cyanobacterial symbionts in lichens (photobionts) that reduce the effects of metal stress (Backor and Fashelt 2008).

Our data indicates that Zn^{2+}, Co^{2+}, and Ni^{2+} at concentrations of 18, 2, and 1 μM respectively did not reduce the viability of *N. punctiforme* within 7 days (Fig. 1b, e, f). Zn^{2+}, Co^{2+}, and Ni^{2+} are all essential trace elements required for different cellular functions for example Zn^{2+} for the activity of all six classes of enzymes and Co^{2+} for the synthesis of cobalamin (vitamin B12) (Singh 1989; Blindauer 2008; Tanioka et al. 2009). The depletion of Zn^{2+}, Co^{2+}, and Ni^{2+} can have drastic effects on cells, inhibiting cell growth by reducing photosynthesis and causing a diversion of ammonia from protein synthesis, which may account for their toxic effects at relatively low concentrations (Daday et al. 1988; Singh 1989; Singh et al. 1992; Cavet et al. 2003; Blindauer 2008; Tanioka et al. 2009). Our data is consistent with previous findings where concentrations of Zn^{2+} ranging from 10 to 100 μM induced a stress response in *Synechocystis* sp. (Ybarra and Webbb 1999). Co^{2+} concentrations exceeding 1 μM inhibited the growth of *Anabaena doliolum* and *Anacystis nidulans* (Singh 1989; Tanioka et al. 2009) while 2 μM of Co^{2+} over 7 days reduced *N. punctiforme* cell growth, but not cell viability (Scholnick and Keren 2006). The viability threshold of 1 μM for Ni^{2+} obtained in our study is higher than previously found where a Ni^{2+} concentration of 0.1 ppm (0.1 μg/mL or 0.06 μM) reduced *Anacystis nidulans* growth over a 120 h period, while 0.5 ppm (0.3 μg/mL or 0.3 μM) of Ni^{2+} reduced *Spirulina platensis* biomass after 168 h (Azeez and Banerjee 1991). Cell growth of *Anabaena cylindrica,* on the other hand, was enhanced by the addition of 0.68 μM Ni^{2+} (Daday et al. 1988).

Cd^{2+} and Cu^{2+} concentrations greater than 0.5 μM caused a reduction in cell viability (Fig. 1a), which is consistent with previous findings for *Synechococcus sp* (Miccadei and Floridi 1993; Gardea-Torresdeya et al. 1998; Ybarra and Webbb 1999). Cu^{2+} concentrations of 1 μM have previously been shown to induce stress responses in *Synechococcus* and reduce cellular oxygen evolution (Ybarra and Webbb 1999). Low concentrations of Cu^{2+} are required as a cofactor for oxygenic electron transfer and in thylakoid plastocyanin (Cavet et al. 2003; Scholnick and Keren 2006). Cu^{2+} toxicity is attributed to its two oxidation states and generation of free radicals (Choudhary et al. 2006). The toxic effects at concentrations of 0.05–0.2 mg/L are associated with an increase in superoxide dismutase activity and a reduction in cellular malondialdehyde and proline levels in *Spirulina platensis* (Choudhary et al. 2006). Previously, Cd^{2+} has been shown to be highly toxic to *Synechococcus* sp. with levels as low as 1 μM inducing a stress response and cellular dysfunction (Miccadei and Floridi 1993; Ybarra and Webbb 1999). Cd^{2+} strongly uncouples oxidative phosphorylation and rapidly incorporates into photosynthetic structures, where it inhibits electron transfer during photosynthesis (Miccadei and Floridi 1993; Ybarra and Webbb 1999).

A key finding of our study was that the mixed metal treatment (0.5 μM Cd^{2+}, 2 μM Co^{2+}, 0.5 μM Cu^{2+}, 500 μM Mn^{2+}, 1 μM Ni^{2+}, and 18 μM Zn^{2+} in BG11) did not cause a reduction in cell viability over the seven-day period. This suggested that the different metals do not have synergistic effects and that *N. punctiforme* was able to discriminate between metal ions and regulate the uptake of each metal individually. This observation is consistent with the need for cells to acquire metals for distinct functions, ensuring trace levels are available as cofactors (Fig. 2) (Cavet et al. 2003; Hantke 2005; Nies 2007; Ma et al. 2009). Exposure of cells to a mixed metal solution containing 18 μM Zn^{2+} together with the addition of either 0.5 μM Cd^{2+}, 0.5 μM Cu^{2+}, 2 μM Co^{2+}, or 1 μM Ni^{2+} reduced *N. punctiforme* cell viability (Fig. 2). In contrast to this, exposure of cells to a mixed metal solution with 18 μM Zn^{2+} and 500 μM Mn^{2+} had no effect on cell viability (Fig. 2). Taken together, this data indicated that the addition of Mn^{2+} (500 μM) reduced the toxic effects of the other metals used in this study. This could be attributed to a cellular exclusion of more toxic metals and uptake by an ion transporter that has a preference for Mn^{2+} (Ma et al. 2009).

Competitive experiments using ^{65}Zn confirmed the presence of interactive metal ion pathways. Co^{2+}, Cu^{2+}, Ni^{2+}, and the mixed metal solution significantly reduced Zn^{2+} uptake after 1 h, where as Cd^{2+} and Mn^{2+} had no significant effect (Fig. 3a). After 72 h, inhibition of Zn^{2+} uptake, relative to Zn^{2+} only, was also observed in the presence of Cd^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and the mixed metal treatments (Fig. 3b). Competition between Zn^{2+} and Cd^{2+} resulted in a fivefold reduction in Zn^{2+} internalization of Zn^{2+} (Fig. 3b). Zn^{2+} and Cd^{2+} have been shown to bind to

same sites in the EPS of Altermonas macleodii subsp fijiensis (Loaec et al. 1997). Our results also indicated that Zn^{2+} uptake in *N. punctiforme* is enhanced by 2 μM of Co^{2+}, which could be attributed to the pH (7.8) favoring Zn^{2+} uptake (Mehta and Gaur 2005). Alternatively, this preferential uptake may be a stress response whereby Co^{2+} is the stressor (Ma et al. 2009). Either way, it appears that *N. punctiforme* is able to efficiently identify and regulate Zn^{2+} uptake in the presence of other metals in solution.

A comparison of the accumulation of each metal was made between single metals and a mixed metal solution (Tables 2 and 3). This indicated that the passive sorption of metals by *N. punctiforme* was competitive and based on the relative concentrations of metals. In contrast, exposure to multiple metals reduced the total cellular sorption of Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, while increasing Cd^{2+} sorption in comparison to single metal exposure. The observation that uptake of a specific metal can be influenced by the presence of a different metal may be explained by different uptake systems varying in their affinity for different metals and the different metals have a range of affinities for most coordinating environments in the order of $Mg^{2+}/Ca^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} \geq Zn^{2+}$ and $Cu^{2+} > Cd^{2+}$ (Mullen et al. 1989; Dupont et al. 2010). In addition, internalized Cd^{2+} and Zn^{2+} levels increased in the presence of multiple metals. The uptake rate of individual or multiple metals, provides insight as to the ability of *N. punctiforme* cells to effectively discriminate and regulate between metals and their extracellular concentration.

In summary, metals including Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} had toxic effects on *N. punctiforme* at different concentrations ranging from 0.5 μM for Cd^{2+} and Cu^{2+} to 500 μM for Mn^{2+}. This may be a result of the cellular requirements and the capacity of different metals to impair cellular processes. The toxic effects of Cd^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} were reduced in the presence of 500 μM Mn^{2+}. Zn^{2+} uptake was elevated in the presence of Co^{2+} but inhibited in the presence of other metals. These findings provide evidence that *N. punctiforme* efficiently discriminates between multiple metals with the uptake of one achieved at the exclusion of another, possibly more toxic metal and that metals can also influence the homeostasis of each other as exemplified by the increase in Zn^{2+} uptake in the presence of Co^{2+}.

Acknowledgments We are grateful to Professor John C. Meeks for technical assistance and helpful discussions.

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